S100B overexpressing mutant mice exhibit prolonged behavioural and biochemical responses towards repeated intermittent binge treatments with MDMA

Anna M. S. Kindlundh-Högb erg, Xiaoqun Zhang and Per Svenningsson

Section of Translational Neuropharmacology, Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden

Abstract

The recreational drug 3,4-methylenedioxy-metamphetamine (MDMA; ‘ecstasy’) enhances serotonin and dopamine transmission. Repeated binge treatment with MDMA (5 mg/kg, 3 times daily, 3 h apart, once per week for 4 wk) was found to increase gene expression of S100B, a neurotrophic factor that modulates neuronal plasticity. Mutant mice overexpressing S100B were investigated to better understand how increased S100B expression may influence MDMA-induced biochemical and behavioural responses. In open-field behaviour, the later MDMA binges decreased rearing and thigmotaxis in S100B mutant mice compared to wild-type mice. In the elevated plus-maze, MDMA increased open-arm entries in both genotypes, but less tolerance to this effect was found in S100B mutant mice. Serotonin transporter (SERT) density was up-regulated in the substantia nigra in S100B mutant mice under baseline conditions. MDMA treatment increased SERT in wild-type mice, but did not further increase it in S100B mutant mice. Dopamine transporter density was down-regulated by MDMA in both genotypes in the striatum. 5-HT1B receptor density and G-protein coupling were higher in MDMA-treated S100B mutant mice than in saline-treated mutant mice and MDMA-treated wild-type mice in the medial globus pallidus. In conclusion, repeated MDMA treatment increases S100B mRNA. Certain explorative and anxiolytic-like behaviours in response to MDMA are potentiated and exhibit less tolerance in mice overexpressing S100B. The genotype-dependent behavioural responses are paralleled by adaptations in the serotonin system. Our data indicate that genetic differences in S100B gene expression may predispose individual differences in the responsivity to repeated intake of MDMA.

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Introduction

The recreational drug 3,4-methylenedioxy-metamphetamine (MDMA; ecstasy) is, after cannabis, currently the second most commonly taken controlled drug in Europe and the USA (Cole and Sumnall, 2003). MDMA consumption causes psychostimulant actions such as euphoria, increased energy and enhanced self-confidence (Morgan, 1998; Parrott, 2002). MDMA can also evoke a series of negative psychiatric symptoms such as depression, impulsivity, hallucinations and reduced cognitive performance (Morgan, 1998; Parrott, 2002). The complex actions of MDMA have divided researchers, with some arguing that MDMA could be used therapeutically against anxiety and post-traumatic stress disorder (Check, 2004), whereas others claim that MDMA is a neurotoxic agent that causes neurodegeneration of monoaminergic neurons and induces psychiatric disease states (Green et al., 2003). MDMA induces a mixture of effects that need to be further evaluated with respect to dosing regimens and time-points of experiment (e.g. during drug exposure, in-between drug exposure or withdrawal...
effects) (Koob and Le Moal, 2001; Kreek et al., 2005; Nestler and Aghajanian, 1997).

Acutely, MDMA potentiates serotonin and dopamine neurotransmission. MDMA is taken up via serotonin (SERT) and dopamine (DAT) transporters and acts intracellularly on the vesicular monoamine transporter (VMAT) to release vesicular stores of neurotransmitters (Green et al., 2003; Gudelsky and Nash, 1996; Han and Gu, 2006; Rothman and Baumann, 2003). The serotonin system plays an important role in sensation seeking, inhibitory control, impulsivity, anxiety, mood and aggression (Jacobs and Fornal, 1999; Linnoila et al., 1983; Winstanley et al., 2004). Natural and drug rewards enhance dopamine transmission in the mesocorticolimbic dopamine pathway (Pontieri et al., 1995; Spanagel and Weiss, 1999), and thereby regulate reward-related associative learning (Di Chiara, 1999; Everitt et al., 1999), reward prediction (Schultz, 1998), incentive salience (Berridge and Robinson, 1998), and behavioural sensitization (Kalivas and Nakamura, 1999; Kalivas et al., 1993).

There is evidence that altered serotonin and dopamine neurotransmission underlie actions of MDMA in mice. In SERT knockout (KO) mice, MDMA does not cause any hyperlocomotion (Bengel et al., 1998). Moreover, pharmacological studies have shown that the 5-HT$_{1A}$ receptor is implicated in MDMA-induced locomotor activity (McCreary et al., 1999; Rempel et al., 1993; Scearce-Levie et al., 1999). A study using 5-HT$_{1B}$ KO mice has shown that stimulatory effects of low to moderate doses of MDMA are dependent upon 5-HT$_{1B}$ receptors (Scearce-Levie et al., 1999). Furthermore, we recently performed a large study examining the effects of MDMA on multiple serotonin receptor genes and found that 5-HT$_{1B}$ receptors were strongly up-regulated in several brain regions (Kindlundh-Högberg et al., 2006). In DAT KO mice MDMA alters hyperactive and preserving behaviours (Powell et al., 2004). However, studies in dopaminergic receptor KO mice have shown preserved ability of MDMA to induce hyperlocomotion in D$_1$, D$_2$ and D$_3$ receptor KO mice (Risbrough et al., 2006).

The molecular pathways that are activated by MDMA remain largely unknown. Animal studies have shown that the glia-derived calcium-binding protein S100B exerts neurotrophic effects on serotonin neurons (Azmitia et al., 1990). The damaging effects of ethanol on serotonin neurons involve a reduction of astroglial production of S100B in the raphe nuclei (Eriksen et al., 2000). Notably, the protective effects of a 5-HT$_{1A}$ agonist on ethanol-treated serotonin neurons seems to be related to the ability of these drugs to release the neurotrophic factor S100B from astrocytes (Eriksen et al., 2002). In addition to 5-HT$_{1A}$ receptor agonists (Azmitia et al., 1995), elevated serotonin (Manev and Manev, 2001), has been shown to increase levels of S100B. There is, in fact, both histological and pharmacological data indicating the presence of serotonin transporters and serotonin receptors in glia (Eriksen et al., 2002; Inazu et al., 2001), that could participate in regulating S100B levels. S100B has also been shown to modulate synaptic plasticity in hippocampus (Nishiyama et al., 2002), and mice overexpressing S100B exhibit an increased axonal sprouting and neurite proliferation in this region (Reeves et al., 1994). Increased plasma or cerebrospinal fluid (CSF) S100B levels have been related to various psychiatric disorders such as Alzheimer’s disease (Griffin et al., 1989, 1998), schizophrenia (Rothermundt et al., 2001, 2004), bipolar mania (Machado-Vieira et al., 2002, 2004), and depression (Arolt et al., 2003). Furthermore, there is recent evidence that variants within the S100B gene predispose to a psychotic subtype of bipolar disorder, possibly via alteration in the levels of S100B gene expression (Roche et al., 2007).

Based on the fact that S100B is induced by serotonergic agents and regulates neuronal plasticity in neuronal circuitries, including the serotonin system, it is feasible that S100B is involved in molecular mechanism(s) underlying the actions of MDMA. We therefore studied the effects of repeated intermittent MDMA administration on S100B gene-transcript expression. Furthermore, to study whether individual variations in S100B expression may affect behavioural and biochemical responses to repeated intermittent MDMA administration, experiments were performed in mutant S100B overexpressing mice and their wild-type (WT) littermates. Specifically, we examined the effects of repeated intermittent MDMA administration on locomotor, explorative and anxiety-like behaviours as well as SERT, DAT and 5-HT$_{1B}$ receptor densities and their coupling to G proteins.

Materials and methods

Animals subjected to MDMA administration

S100B overexpressing mutant and WT male mice backcrossed on a C57/B16 background (aged 4–6 months; weighing 28±3 g) were used. The choice of age of the mice was based on a previous report demonstrating an important role of 5-HT$_{1B}$ receptors in MDMA actions by usage of mutant 5-HT$_{1B}$ receptor KO mice (Scearce-Levie et al., 1999). Mice were housed in air-conditioned rooms (12-h dark/light cycle, lights on 07:00 hours) at 20 °C with humidity of 53%. The
experiments were in agreement with international guidelines on the ethical use of animals and are approved by the local ethics committee. The S100B overexpressing mutant mice used in the present study exhibit an overexpression of the S100B gene which is cell type- and tissue-appropriate. This was reported in a previous publication (Reeves et al., 1994) and confirmed in the present study (Figure 1). This was achieved by the transgene expression of a 8.9-kb murine S100b genomic clone. Furthermore, the genetic information included in this sequence is not only sufficient for proper localization of the S100B gene but also for the regulation of the S100B gene at least by MDMA (Figure 2). This model is therefore appropriate for studies of genetic overexpression of S100B in relation to effects of MDMA. WT and mutant mice were bred to generate equal amounts of each genotype, which were determined by PCR. Based on the PCR genotyping, WT and S100B mutant mice were originally separated into two MDMA-treated groups and two saline-treated control groups (originally n = 7–8 per group). However, during the confirmatory in-situ hybridization, it became obvious that mice were not correctly genotyped. Therefore the final group numbers were as follows: WT mice receiving MDMA (ecstasy) (WE, n = 6); Mutant mice receiving MDMA (ecstasy) (ME, n = 9); WT mice receiving saline serving as controls (WC, n = 6); Mutant mice receiving saline serving as mutant controls (MC, n = 9). The mice received four repeated MDMA or saline binge treatments every 7th day and each treatment consisted of three intraperitoneal injections; MDMA-treated mice received 3 × 5 mg MDMA/kg and controls 3 × saline.

Human MDMA abusers usually use single dosages of 50–160 mg. Novice users generally take a single ecstasy tablet, regular users typically take 2–3 tablets, whereas the most experienced users may take 10–25 tablets in a single session (Parrott, 2005). Therefore, animal models for MDMA abuse usually attempt to mirror abuse in humans in terms of administered binges (e.g. O’Shea et al., 2001; Scearce-Levie et al., 1999). Using the inter-species scaling technique:

\[
\text{dose}_{\text{human}} = \text{dose}_{\text{animal}} \times (\text{weight}_{\text{human}} / \text{weight}_{\text{animal}})^{0.7}
\]

doses administered to the mice in this study correspond to the lower doses used in a tablet by humans (Mordenti and Chappell, 1989).

**Behavioural studies**

The open-field behaviour was recorded directly after each binge treatment. Four identical square open-field

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**Figure 1.** (a) Photograph from an agarose gel from a PCR reaction using tail DNA from WT and S100B mutant mice showing the presence of the construct that overexpresses S100B in S100B mutant mice. (b) The gene expression of S100B in a WT mouse. (c) The gene expression of S100B in a mutant mouse overexpressing this gene. WT, wild-type mouse; MUT, S100B overexpressing mutant mouse. Scale bar, 3 mm.

**Figure 2.** Effects upon S100B mRNA levels in the cortex following repeated intermittent administration of saline or MDMA (3 × 5 mg MDMA/kg) for 4 wk in (a) wild-type and (b) mutant mice, respectively. Statistics: unpaired Student’s t test. Significance levels: * p < 0.05, ** p < 0.01.
arenas (outside size: 650 × 650 × 325 mm; inside size: 500 × 500 × 225 mm) were used. The arenas were equipped with two rows of photocells, sensitive to infrared light, placed 20 mm and 50 mm above the floor. The photocells were spaced 31 mm apart, and the last photocell in a row was spaced 17.5 mm from the wall. The open field was enclosed in a ventilated, sound-attenuated box (Kungsbacka Mät och Reglerteknik AB, Fjärås, Sweden). Recordings were conducted in the dark, between 19:15 and 22:15 hours. The numbers of photocell beam interruptions were recorded on a computer. The variables recorded were: total (or horizontal) activity (HA) (i.e. all interruptions of photobeams in the lower rows); locomotor (ambulatory) activity (L) (i.e. successive interruptions of photocells in the lower rows); rearing activity (RA) (i.e. all interruptions of photobeams in the upper rows); peripheral activity (PA) (i.e. interruptions of photobeams spaced 17.5 mm from the wall in the lower rows); L and PA are presented as percent of total activity. To avoid biased results of general hyperactivity, the behaviours in the elevated plus-maze were measured 24 h after each binge administration. An automated elevated plus-maze instrument equipped with infrared beams (TSE Systems GmbH, Bad Ansgons, Germany) measured time in open and closed arms as well number of transitions between arms. The elevated plus-maze apparatus consisted of two opposite open arms (5 cm wide × 30 cm long) and two opposite closed arms (5 cm wide × 15 cm high × 30 cm long). The ledges on the open arms were 0.3 cm high. The apparatus was placed on a stand 54 cm above the floor. Animals were placed in the centre of the maze with the head oriented towards the open arm and the time spent in the different arms was recorded for 5 min. The number of entries in the different arms was measured as an index of exploratory behaviours. Between tests, the maze was cleaned and dried.

**Tissue preparation**

All animals were sacrificed by decapitation 1 h after the last intraperitoneal injection. The brains were rapidly removed at −20 °C to −30 °C and stored at −80 °C. The brains were cut in a cryostat at −19 °C into 12-μm coronal sections.

**In-vitro autoradiography of DAT, SERT and 5-HT<sub>1B</sub> Receptors**

Slide-mounted sections for detection of DAT and SERT were preincubated in 50 mM Tris–HCl/120 mM NaCl (pH 7.5) for 20 min. Incubation in binding buffer [50 mM Tris–HCl/120 mM NaCl (pH 7.5)] was conducted with 50 pM [<sup>125</sup>I]RTI-55 (PerkinElmer Life Sciences, Boston, MA, USA) for 60 min in the presence of either 1 μM fluoxetine (selective serotonin reuptake inhibitor) to label DAT or 1 μM nomifensine (selective dopamine reuptake inhibitor) to label SERT. For non-specific binding, 100 μM nomifensine or fluoxetine were added to the assay. The slides were washed for 2 × 10 s in ice-cold binding buffer, rapidly dipped in deionized water and dried.

The brain sections were pre-incubated in buffer that dilutes residual MDMA and reduces the competition of MDMA with the transporter radioligand. Furthermore, sections from mice administered single and repeated MDMA binge treatments were simultaneously processed. As detailed below, no changes in transporter levels were found in mice after a single binge treatment of MDMA, it is therefore unlikely that any residual MDMA would cause the significant effects observed after repeated MDMA binge treatments.

To label 5-HT<sub>1B</sub> receptors, sections were preincubated at room temperature with 170 mM Tris/150 mM NaCl (pH 7.4) for 10 min, incubated for 2 h in the same buffer supplemented with 30 μM isoprenaline to block β-adrenergic receptor sites and 12 pM [<sup>35</sup>S]-iodocyanopindolol (5-HT<sub>1B</sub> receptor antagonist) in the absence (total binding) or presence (non-specific binding) of 10 μM serotonin (Sari et al., 1999).

**Guanosine 5′-O-3-thiotriphosphate (GTP<sub>S</sub>) binding**

In order to analyse 5-HT<sub>1B</sub> receptor-mediated stimulation of GTP<sub>S</sub>, all slides were subjected to a stabilization phase for 10 min, a preincubation phase for 30 min, and an incubation phase for 120 min at 25 °C. The stabilization assay (buffer 1) contained 50 mM Tris–HCl, 3 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 100 mM NaCl, and 1 mM DTT. For the preincubation assay (buffer 2) 2 mM GDP and 9.5 mM U/ml adenosine deaminase were also included. Incubation of slides for estimation of basal levels was conducted in buffer 2, but in the presence of 0.5% BSA and 0.04 mM [γ<sup>32</sup>P]-GTP. The 5-HT<sub>1B</sub> receptor agonist CP93129 at 1.0 μM was included in the incubation assay in order to investigate the stimulation of GTP<sub>S</sub> binding. The slides were washed twice at 4 °C for 15 minutes in buffer 1, of which 10 U/ml adenosine deaminase was also included. Finally all slides were dipped in water at 4 °C.

**In-situ hybridization of S100B and 5-HT<sub>1B</sub> receptor mRNA levels**

An antisense <sup>35</sup>S-labelled cRNA probe was prepared from [γ<sup>35</sup>S]UTP by in-vitro transcription from a cDNA
clone corresponding to a fragment of the gene encoding S100B. Sections were hybridized with this probe, washed and dried as described (Svenningsson et al., 1997). A synthetic oligonucleotide probe complementary to nucleotides 745–790 in the mouse 5-HT1B receptor mRNA sequence (Maroteaux et al., 1992) was synthesized by Cybergene, Sweden. Substance P mRNA was detected by a probe corresponding to nucleotides 124–171 (Krause et al., 1987). Preproenkephalin mRNA was analysed by an antisense probe towards nucleotides 388–435 (Yoshikawa et al., 1984). The oligonucleotide probe was 3’-end labelled with deoxyadenosine 5’-[α-35S]thiotriphosphate and used for in-situ hybridization as previously described (Svenningsson et al., 1995).

**Film exposure and data analysis**

Labelled sections and plastic standards [125I] microscales (2.2–160 nCi/mg) (GE Healthcare, Uppsala, Sweden), or [3H] microscales (40.1–1112.8 nCi/g) for re-calculating [35S] (GE Healthcare, Uppsala, Sweden) were mounted and exposed to autoradiographic films (Kodak BioMax MR, Merck Eurolab, Sweden) in X-ray cassettes at −20 °C for 2–7 d ([125I]), or for 14 d ([35S]/[3H]). The films were manually developed (Kodak D19, Unifix). Autoradiograms were digitized using a Dia-Scanner (Epson Perfection 4870 Photo). Based on co-exposed standards optical densities were converted to amol/mg wet weight or fmol/mg wet weight using Scion Image for Windows, version alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA). Brain regions were identified with a mouse brain atlas (Paxinos and Watson, 1997).

**Autoradiograms**

Representative autoradiograms of quantitative in-vitro autoradiography and in-situ hybridization are shown in Supplementary Figure S1. Abbreviations at bregma B ± 1.1 mm of DAT and SERT density, as well as 5-HT1B receptor mRNA: CPu, caudate putamen (dorsolateral, ventrolateral, medial); at B = 0.1 and −0.3 of 5-HT1B: VP/LGP, ventral pallidum/lateral globus pallidus; at B = −1.3 of 5-HT1B: MGP, medial globus pallidus (also termed entopeduncular nucleus); at B = −3.2 of SERT and 5-HT1B: SNR, substantia nigra pars reticulata.

**Neuronal and glial cell density**

Nissl staining was performed in order to confirm the number of neuronal and glial cells in the ventral striatum of mice. Prior to 3 min of staining in 0.5% Cresyl Violet, the tissue sections were dehydrated and hydrated into graded ethanol concentrations (75, 95, 100, 100, 95, 75%, H2O for 1 min each). The stained sections were washed in 100% ethanol solution (1 min) and cleaned for 30 min in Xylene. Slides were mounted (Mountex, Histolab; Gothenburg, Sweden) and cell numbers assessed in a light microscopy (Nikon Eclipse, E600) using the software programs Nis Elements (F2.2) and WCIF Image J (1.37a).

**Statistical analysis**

Behavioural results were evaluated as follows: the parametric two-way ANOVA repeated measurement followed by Fisher’s PLSD test was applied on HA and RA as well as results obtained from elevated plus-maze experiments. The non-parametric Friedman’s repeated measurements followed by Dunn’s multiple comparison test was performed on ratios of behavioural measurements (i.e L/HA, PA/HA). Biochemical results were evaluated as follows: two-tailed unpaired Student’s t test was used for analyses of the expression of S100B mRNA in WT and mutant mice, respectively. Two-way ANOVA followed by Fisher’s PLSD for post-hoc analyses were applied for comparisons between the four independent groups [WC, wild-type saline-treated control; WE, wild-type MDMA (ecstasy); MC, mutant control; ME, mutant MDMA (ecstasy)] with respect to all biochemical analyses of receptors and transporters.

**Results**

**MDMA-induced effects on S100B mRNA levels**

Repeated intermittent MDMA administration caused a significant increase in S100B mRNA levels of the cortex in both WT mice and mutant S100B over-expressing mice (Figure 2).

**Effects of repeated intermittent MDMA binge treatments on open-field activity in WT and S100B mutant mice**

Total RA was similar in WC, MC, WE and ME mice in week 1 \(F(3,28)=0.82, p=0.4916\). In contrast, in week 2 \(F(3,27)=3.39, p=0.0324\) and in week 4 \(F(3,27)=5.15, p<0.0061\), RA was significantly down-regulated in ME mice compared to both MC and WE mice (Figure 3). This difference in RA was apparent after 10 min and lasted for 45 min. HA was significantly increased with no genotype differences by each weekly MDMA binge treatment (Figure 4a; week 1 \(F(3,28)=44.06, p<0.0001\), week 2 \(F(3,27)=33.41,
Figure 3. Total rearing activity (RA) following repeated intermittent administration of saline or MDMA (3 × 5 mg MDMA/kg·d). WC, wild-type saline control mice (n = 6); MC, mutant saline control mice (n = 9); WE, wild-type ecstasy (MDMA) mice (n = 6); ME, mutant ecstasy (MDMA) mice (n = 9). Statistics: two-way ANOVA repeated measurements followed by Fisher’s PLSD test. Significance levels for WE vs. WC: * p < 0.05, ** p < 0.01, *** p < 0.001. For WE vs. ME: * p < 0.01, ++ p < 0.001.

Figure 4. (a) Total horizontal activity (HA), (b) locomotor activity vs. total horizontal activity (L/HA), (c) peripheral activity vs. total horizontal activity (PA/HA) following repeated intermittent administration of saline or MDMA (3 × 5 mg MDMA/kg·d). WC, wild-type saline control mice (n = 6); MC, mutant saline control mice (n = 9); WE, wild-type ecstasy (MDMA) mice (n = 6); ME, mutant ecstasy (MDMA) mice (n = 9). Statistics: (a) parametric two-way ANOVA repeated measurements followed by Fisher’s PLSD test; (b) and (c) non-parametric Friedman’s repeated measurements of ratios followed by Dunn’s test. Significance levels for WE/ME vs. WC/MC, respectively: * p < 0.05, ** p < 0.01, *** p < 0.001. For WE vs. ME: + p < 0.05.
Effects of repeated intermittent MDMA binge treatments in the elevated plus-maze in WT and S100B mutant mice

Open-arm entries divided by total arm entries (OA/TA) was shown to be significantly different between groups according to two-way ANOVA repeated measurements by treatment [F(3, 23) = 3.62, p = 0.0282] and time (over the weeks) [F(2, 46) = 13.96, p < 0.0001], but there were no interactions between treatment and time [F(6, 46) = 1.95, p = 0.0921] (Figure 5a). Post-hoc analyses using Fisher’s PLSD post-hoc test showed that percentage of OA/TA was increased in both WE and ME mice compared to WC and MC mice, after the first MDMA binge treatment. An increase in OA/TA was also observed for ME, but not WE mice, after the second binge treatment. Post-hoc analyses investigating alterations within groups by time showed no alterations in any of the saline-treated mice. However, in WE mice, percentage OA/TA was significantly decreased over time, both between weeks 1 and 2, and weeks 1 and 3. The OA/TA in ME mice was decreased between weeks 1 and 2, but not between weeks 1 and 3. There was a significant alteration in total activity by time (weeks) [F(2, 46) = 16.57, p < 0.0001], but no significant alteration by treatment [F(3, 23) = 2.60, p = 0.0768] or any interaction by time in treatment [F(6, 46) = 0.96, p = 0.4647] (Figure 5b). Open-arm duration, in terms of percentage open-arm time divided by total arm time (OAT/TAT) was significant by treatment [F(3, 23) = 3.46, p = 0.0328] and time [F(2, 46) = 13.49, p < 0.0001], but not the interaction of these measures [F(6, 46) = 1.09, p = 0.3813]. Post-hoc analysis showed that in week 3 the percentage OA duration was increased for ME compared to MC mice (data not shown).

Repeated intermittent MDMA-induced effects upon protein density, mRNA expression, and 5HT1B receptor-induced GTPγS activity in WT and S100B mutant mice

No difference in DAT density was found between WC and MC mice (Table 1a, Figure 6). However, MDMA caused a significant reduction of DAT density in CPu in both genotypes (Table 1a, Figure 6). SERT density was significantly higher in MC than WE mice in SNR, but not in CPu (Table 1a, Figure 6). In SNR an increase in SERT density was also detected in WE mice. No significant difference in SERT between MC and ME mice was found (Table 1a, Figure 6). The 5-HT1B receptor mRNA expression levels were significantly increased in the CPu in WE and ME mice compared to WC and MC mice, respectively (Table 1b, Figure 7a).
The 5-HT$_{1B}$ receptor binding was significantly increased in MGP in ME mice compared to WE and MC mice, respectively (Table 1b, Figure 7b). The increased 5-HT$_{1B}$ receptor binding in MGP was paralleled by increased GTP$_{S}$ binding in response to the agonist CP93129 in ME mice compared to WE and MC.
Table 1b. Effects of repeated intermittent MDMA administration at a dose of 3×5 mg/kg given every 7th day for 4 wk, upon the 5-HT1B receptor mRNA level, 5HT1B receptor density, and 5HT1B GTPγS activity

<table>
<thead>
<tr>
<th>Brain region</th>
<th>WC (n = 6)</th>
<th>MC (n = 9)</th>
<th>WE (n = 6)</th>
<th>ME (n = 9)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± S.E.M.</td>
<td>mean ± S.E.M.</td>
<td>mean ± S.E.M.</td>
<td>mean ± S.E.M.</td>
<td>F statistics</td>
</tr>
<tr>
<td>5-HT1B receptor mRNA</td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
<td>F(3, 23) = 6.27</td>
</tr>
<tr>
<td>Bregma +1.1</td>
<td>100.0 ± 10</td>
<td>85.6 ± 9</td>
<td>153.1 ± 16*</td>
<td>139.8 ± 13**</td>
<td></td>
</tr>
<tr>
<td>CPu</td>
<td>5533.0 ± 623</td>
<td>4759.6 ± 291</td>
<td>4856.0 ± 250</td>
<td>5571.3 ± 367</td>
<td>F(3, 26) = 1.28</td>
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<tr>
<td>5-HT1B receptor binding</td>
<td>(amol/mg)</td>
<td>(amol/mg)</td>
<td>(amol/mg)</td>
<td>(amol/mg)</td>
<td>F(3, 26) = 0.19</td>
</tr>
<tr>
<td>Bregma –0.1</td>
<td>4657.3 ± 588</td>
<td>4769.3 ± 319</td>
<td>5167.0 ± 638</td>
<td>5020.1 ± 502</td>
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</tr>
<tr>
<td>VP and LGP</td>
<td>780.2 ± 100</td>
<td>1065.0 ± 162</td>
<td>1028.9 ± 211</td>
<td>1659.1 ± 246*</td>
<td></td>
</tr>
<tr>
<td>Bregma –3.2</td>
<td>6451.4 ± 852</td>
<td>6938.3 ± 419</td>
<td>7042.2 ± 426</td>
<td>8810.8 ± 387**</td>
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<tr>
<td>SN</td>
<td>100.0 ± 15</td>
<td>95.1 ± 12</td>
<td>84.9 ± 14</td>
<td>125.0 ± 17</td>
<td>F(3, 26) = 1.44</td>
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<td>5-HT1B GTPγS binding</td>
<td>(amol/mg)</td>
<td>(amol/mg)</td>
<td>(amol/mg)</td>
<td>(amol/mg)</td>
<td>F(3, 26) = 0.92</td>
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<td>Bregma –0.1</td>
<td>100.0 ± 15</td>
<td>103.1 ± 4</td>
<td>101.7 ± 6</td>
<td>120.0 ± 4**</td>
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<tr>
<td>VP and LGP</td>
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<td>117.8 ± 10</td>
<td>126.8 ± 22</td>
<td>123.1 ± 17</td>
<td>F(3, 25) = 0.49</td>
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</table>

WC, Wild-type saline-treated control; MC, mutant control; WE, wild-type MDMA (ecstasy); ME, mutant MDMA (ecstasy); CPu, caudate putamen; VP, ventral pallidum; LGP, lateral globus pallidus; MGP, medial globus pallidus; SN, substantia nigra. Significance levels: Fisher’s PLSD test due to MDMA effect compared to saline controls: * p < 0.05, ** p < 0.01. Significance due to a mutant effect among MDMA-treated mice (WE vs. ME) using Fisher’s PLSD test: + p < 0.05.

Figure 7. Autoradiograms illustrating the effects of repeated intermittent administration of saline or MDMA (3×5 mg MDMA/kg given every 7th day for 4 wk) upon (a) 5-HT1B receptor mRNA levels, (b) 5-HT1B receptor binding by [125I]iodocyanopindolol, and (c) 5-HT1B GTPγS stimulation. Scale bar, 3 mm.
mice (Table 1b, Figure 7c). Repeated intermittent MDMA administration caused a significant increase of substance P mRNA levels in WE and ME mice compared to the saline-treated mice, while mRNA levels of preproenkephalin remained unchanged (Table 1c).

To study whether the biochemical effects after MDMA were dependent upon single or repeated binge treatments, we injected a set of mice with a single MDMA binge treatment. There were no significant alterations of DAT and SERT densities in any of the investigated regions following this single binge administration of MDMA (Table 2a). The 5-HT1B receptor density was significantly decreased in VP/LGP at doses of 3 × 5 and 3 × 10 mg MDMA/kg, as well as in LGP at 3 × 10 mg MDMA/kg (Table 2b). In the MGP, the 5-HT1B receptor density was significantly increased by all three MDMA dose regimens. Acute MDMA (3 × 5 and 3 × 10 mg/kg) increased 5-HT1B receptor mRNA in CPu (Table 2b).

**Neuronal and glial cell density**

Since high doses of MDMA have been shown to exert toxic actions, we examined the effects of MDMA on neuronal and glial cell densities. Long-term repeated binge treatments of MDMA to WT and S100B mutant mice did not cause any significant alterations of neuronal [F(3, 18) = 0.63, p = 0.6054] and glial [F(3, 18) = 0.47, p = 0.7048] cell density in the striatum (Figure 8).

**Discussion**

The present study shows that MDMA increases S100B mRNA expression in WT and mutant S100B mice (Table 1b, Figure 7c). Repeated intermittent MDMA administration caused a significant increase of substance P mRNA levels in WE and ME mice compared to the saline-treated mice, while mRNA levels of preproenkephalin remained unchanged (Table 1c).

<table>
<thead>
<tr>
<th>Table 1c. Effects of repeated intermittent MDMA administration at a dose of 3 × 5 mg/kg given every 7th day for 4 wk upon enkephaline mRNA and substance P mRNA levels in the caudate putamen (CPu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain region</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Enkephaline mRNA</td>
</tr>
<tr>
<td>Bregma +1.1</td>
</tr>
<tr>
<td>CPu</td>
</tr>
<tr>
<td>Substance P mRNA</td>
</tr>
<tr>
<td>CPu</td>
</tr>
</tbody>
</table>

WC, Wild-type saline-treated control; MC, mutant control; WE, wild-type MDMA (ecstasy); ME, mutant MDMA (ecstasy). Significance levels: Fisher’s PLSD test due to MDMA effect compared to saline controls: * p < 0.05.

<table>
<thead>
<tr>
<th>Table 2a. Acute MDMA effects upon densities of dopamine (DAT) and serotonin (SERT) transporters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain region</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>DAT</td>
</tr>
<tr>
<td>Bregma +1.1</td>
</tr>
<tr>
<td>CPu dorsolateral</td>
</tr>
<tr>
<td>CPu lateral</td>
</tr>
<tr>
<td>CPu medial</td>
</tr>
<tr>
<td>SERT</td>
</tr>
<tr>
<td>Bregma −3.2</td>
</tr>
<tr>
<td>SN</td>
</tr>
</tbody>
</table>

CPu, Caudate putamen; SN, substantia nigra.
overexpressing mice. The effect in S100B mutant mice indicates that the transgene construct includes a promoter region that is responsive to transcriptional regulation by MDMA.

To examine explorative and anxiolytic-like behaviours, studies were conducted in the open-field and elevated plus-maze. Previous studies have shown reduced rearing following MDMA administration in mice (Maldonado and Navarro, 2000; Scearce-Levie et al., 1999). Here, we found no significant effect of MDMA on rearing in WT mice. However, S100B mutant mice increased forward locomotion in response to the first binge treatment. However, only S100B mutant mice showed sustained increase in forward locomotion in subsequent binge treatments, indicating less tolerance compared to their WT littermates. It could not be excluded that the lack of effects among WT mice on RA and the latter binges of forward locomotion could partly be explained by a relatively small number of subjects per group.

MDMA-treated S100B mutant mice developed less thigmotaxis compared to the MDMA-treated WT mice. This reduction in thigmotaxis indicates that MDMA causes some anxiolytic-like actions in S100B mutant mice that were not found in the WT littermates. The effects of MDMA in the elevated plus-maze were measured 24 h after each binge treatment to avoid disturbances of hyperactivity. Using this short-time withdrawal protocol, we found that MDMA induced anxiolytic-like effects reflected by increased proportion of open-arm entries in the elevated plus-maze in both WT and S100B mutant mice after the first binge. These results suggest that S100B mutant mice are more sensitive to the anxiolytic-like effects of MDMA compared to WT mice.

### Table 2b. Acute MDMA effects upon the 5-HT$_{1B}$ receptor density and mRNA levels

<table>
<thead>
<tr>
<th>Bregma</th>
<th>Controls (n=6)</th>
<th>MDMA Low (n=6)</th>
<th>MDMA High (n=6)</th>
<th>MDMA vs. High (n=6)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP/LGP</td>
<td>(amol/mg)</td>
<td>(amol/mg)</td>
<td>(amol/mg)</td>
<td>(amol/mg)</td>
<td>F(3, 20) = 6.74, p = 0.0025</td>
</tr>
<tr>
<td>0.1</td>
<td>5698.7±460</td>
<td>4508.6±579</td>
<td>3661.3±361**</td>
<td>3044.2±327***</td>
<td>5.55, p = 0.0061</td>
</tr>
<tr>
<td>LGP</td>
<td>4730.7±743</td>
<td>4568.9±480</td>
<td>3238.8±314</td>
<td>2320.9±209**</td>
<td>6.33, p = 0.0034</td>
</tr>
<tr>
<td>MGP</td>
<td>505.2±70</td>
<td>1020.6±83**</td>
<td>1000.2±93**</td>
<td>869.1±125*</td>
<td>4.21, p = 0.0192</td>
</tr>
<tr>
<td>SN</td>
<td>7492.4±1166</td>
<td>5848.2±723</td>
<td>6301.1±825</td>
<td>6598.4±466</td>
<td>0.69, p = 0.5701</td>
</tr>
</tbody>
</table>

VP, ventral pallidum; LGP, lateral globus pallidus; MGP, medial globus pallidus; SN, substantia nigra; CPu, caudate putamen. Significance levels: Fisher’s PLSD test: *p < 0.05, **p < 0.01, ***p < 0.001.

![Figure 8. Relative neuronal and glial cell density in percent related to controls after repeated intermittent administration of saline or MDMA (3×5 mg MDMA/kg.d) for 4 wk. WC, wild-type saline control mice; MC, mutant saline control mice; WE, wild-type ecstasy (MDMA) mice; ME, mutant ecstasy (MDMA) mice.](http://ijnp.oxfordjournals.org/)

MDMA-treated S100B mutant mice developed less thigmotaxis compared to the MDMA-treated WT mice. This reduction in thigmotaxis indicates that MDMA causes some anxiolytic-like actions in S100B mutant mice that were not found in the WT littermates. The effects of MDMA in the elevated plus-maze were measured 24 h after each binge treatment to avoid disturbances of hyperactivity. Using this short-time withdrawal protocol, we found that MDMA induced anxiolytic-like effects reflected by increased proportion of open-arm entries in the elevated plus-maze in both WT and S100B mutant mice after the first binge. These results suggest that S100B mutant mice are more sensitive to the anxiolytic-like effects of MDMA compared to WT mice.
binge treatment. The anxiolytic-like effect of MDMA in the elevated plus-maze showed less tolerance in S100B mutant mice compared to their WT littermates.

The primary and immediate action of MDMA is to increase extracellular levels of serotonin and dopamine levels, which is known to be followed by decline or even depletion of these neurotransmitters (Morton, 2005). Low levels of monoamines in between administration of repeated MDMA challenges may precipitate depression among abusers and is known as the ‘mid-week blues’ (Morton, 2005). Since increased S100B concentrations have been ascribed neurotrophic properties and promote neural plasticity in neurons and glial cells (Rothermundt et al., 2003), it is possible that S100B counteracts tolerance, and even potentiates, certain explorative and anxiolytic-like behaviours after repeated MDMA administration via altered neuronal plasticity and neurotrophic actions on the monoamine system.

Pronounced changes in 5-HT1B receptor levels were found in response to repeated intermittent MDMA binge administrations. 5-HT1B receptor mRNA levels were significantly up-regulated in CPu in both WT and S100B mutant mice treated with MDMA. S100B mutant, but not WT, mice receiving MDMA, also exhibited a significant increase in the levels of 5-HT1B receptor-binding sites in MGP. These 5-HT1B receptors are probably transported from CPu and localized at axon terminals in MGP, where they serve as hetero-receptors (Boschert et al., 1994; Maroteaux et al., 1992). In MGP, 5-HT1B receptor agonist-induced [35S]GTP was significantly increased in MDMA-treated S100B mutant mice.

Thus, our data indicate a particularly important role of 5-HT1B receptors located in the so-called direct striatal output pathway in modulating long-term effects of MDMA. This direct pathway is comprised of striatal neurons terminating in either MGP or SNR and contains the neuropeptide substance P (Gerfen et al., 1990). About 50% of the striatal neurons belong to the direct pathway and the other 50% of the striatal neurons to the indirect pathway that project to LGP and contain the neuropeptide enkephalin. Our data on 5-HT1B receptor up-regulation indicates that the direct pathway is affected by repeated intermittent MDMA administration. A regulation of the direct pathway by MDMA is further supported by the fact that substance P mRNA levels were increased in WE and ME mice. This is interesting given the vast evidence that molecular adaptations in the direct pathway occurs in response to repeated treatment with other psycho-stimulants, including cocaine and d-amphetamine (Nestler, 2005).

To study whether the observed effects on 5-HT1B receptor levels by repeated intermittent binge treatments with MDMA were dependent on a single or repeated binge treatment, we also injected a set of mice with a single binge treatment. The 5-HT1B receptor mRNA level was up-regulated both after a single and repeated MDMA binge treatments. 5-HT1B receptor levels in MGP were increased in response to a single binge treatment. However, in WT mice, the increased 5-HT1B receptor density in MGP was not sustained. In contrast, in S100B mutant mice, there was a persistent increase in 5-HT1B receptor density in MGP. This indicates that the normalization of 5-HT1B receptor binding following repeated treatments in WT mice could involve post-transcriptional mechanisms which are differently regulated in S100B mutant mice. There was a significant reduction of 5-HT1B Receptor density in LGP in mice administrated a single MDMA binge treatment, that was not found in the chronically MDMA-treated mice regardless of genotype. These data indicate that there are changes in 5-HT1B Receptor level also in striatopallidal neurons that are normalized upon repeated treatment.

Another S100 family protein, p11, affects 5-HT1B receptor function by directly interacting with the receptor (Svenningsson et al., 2006). Indeed, p11 increases localization of 5-HT1B receptors at the cell surface. Moreover, p11 KO mice have reduced responsiveness to 5-HT1B receptor stimulation and to behavioural reactions to an antidepressant. Although we now report a general increase of 5-HT1B Receptor binding in S100B mutant mice, we have, at the moment, no evidence that S100B directly interacts with 5-HT1B receptors.

Regarding effects of MDMA on SERT and DAT proteins, a genotype effect was found on the levels of SERT in SNR in saline-treated control animals. This increase in SERT density in S100B mutant mice can be interpreted as an increased serotonin innervation of this area in S100B mutant mice under baseline conditions. Since MDMA acts on SERT and SERT KO mice are insensitive to MDMA (Bengel et al., 1998), it could be argued that increased SERT in S100B mutant mice under baseline conditions would cause an increased responsiveness towards MDMA. This may, indeed, participate in the increased effects of MDMA in S100B mutant mice, but is unlikely to be the sole explanation since the effects of MDMA in these mice were seen mostly after chronic, but not acute, MDMA treatment. Up-regulated SERT binding in SNR was found following long-term intermittent MDMA binge administrations in WT mice and there was a trend for MDMA-treated S100B mutant mice to have further
increased SERT in SNR. These increases, and the lack of effect of MDMA on SERT in numerous other regions, argue against any neurotoxic effects of MDMA on serotonin neurons using the current treatment protocol. In contrast, DAT was significantly down-regulated by MDMA in both WT and mutant S100B mice in CPu. Down-regulation of DAT density in our long-term study, either reflects loss of nigrostriatal dopamine neurons or reduced numbers of DAT, which is in line with postulated MDMA-induced dopaminergic neurotoxicity in mice (Colado et al., 2001). In any case, this down-regulation of DAT was not altered by S100B overexpression. Since the present results show that the mice exhibit persistent enhanced horizontal activity, the reduced number of DAT may not be functionally significant. There were no significant differences in SERT and DAT densities in any of the investigated regions in WT mice treated with a single MDMA binge treatment, so all the above-mentioned effects depend on genotype or the repeated intermittent MDMA binge treatment paradigm.

Moreover, repeated intermittent MDMA administration did not affect the neuronal and glial cell density in any of the genotypes at the doses used in the present study. This indicates that changes in receptor protein densities and gene-transcript expressions are not caused by cell loss or neurotoxicity. The present study demonstrates for the first time an involvement of S100B in modulating mechanisms underlying plastic adaptations in response to repeated intermittent MDMA administration. In conclusion, the present study demonstrates for the first time that: (i) MDMA increases S100B gene expression levels; (ii) MDMA affects the levels of a glial-derived gene (i.e S100B); (iii) certain MDMA-mediated effects on explorative behaviours, including rearing, are potentiated in mutant S100B overexpressing mice; (iv) mutant S100B overexpressing mice appear to have a potentiated responsivity and develop less tolerance to anxiolytic-like effects of MDMA; (v) MDMA-mediated up-regulation of 5-HT_{1B} receptor mRNA, protein and G-protein coupling is increased in the direct striatal output pathway via a mechanism that is enhanced in mutant S100B mice; (vi) SERT is up-regulated in SNR in S100B mutant mice under baseline conditions. Genetic variability in the S100B gene expression may predispose individual differences in responsivity to MDMA.

Note

Supplementary material accompanies this paper on the Journal’s website (http://journals.cambridge.org).

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Statement of Interest

None.

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